

Proteomic analysis reveals Hrs ubiquitin-interacting motif-mediated ubiquitin signaling in multiple cellular processes

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Keywords

endocytic trafficking; Hrs; *in vitro* expression cloning; ubiquitination; ubiquitin-interacting motif

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(Received 26 June 2008, revised 19 October 2008, accepted 24 October 2008)

doi:10.1111/j.1742-4658.2008.06760.x

Despite the critical importance of protein ubiquitination in the regulation of diverse cellular processes, the molecular mechanisms by which cells recognize and transmit ubiquitin signals remain poorly understood. The endosomal sorting machinery component hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) contains a ubiquitin-interacting motif (UIM), which is believed to bind ubiquitinated membrane cargo proteins and mediate their sorting to the lysosomal degradation pathway. To gain insight into the role of Hrs UIM-mediated ubiquitin signaling in cells, we performed a proteomic screen for Hrs UIM-interacting ubiquitinated proteins in human brain by using an *in vitro* expression cloning screening approach. We have identified 48 ubiquitinated proteins that are specifically recognized by the UIM domain of Hrs. Among them, 12 are membrane proteins that are likely to be Hrs cargo proteins, and four are membrane protein-associated adaptor proteins whose ubiquitination may act as a signal to target their associated membrane cargo for Hrs-mediated endosomal sorting. Other classes of the identified proteins include components of the vesicular trafficking machinery, cell signaling molecules, proteins associated with the cytoskeleton and cytoskeleton-dependent transport, and enzymes involved in ubiquitination and metabolism, suggesting the involvement of Hrs UIM-mediated ubiquitin signaling in the regulation of multiple cellular processes. We have characterized the ubiquitination of two identified proteins, Munc18-1 and Hsc70, and their interaction with Hrs UIM, and provided functional evidence supporting a role for Hsc70 in the regulation of Hrs-mediated endosome-to-lysosome trafficking.

Ubiquitination is a post-translational modification in which the 76 amino acid polypeptide ubiquitin is covalently attached to a lysine residue(s) of substrate proteins [1]. Proteins can be either monoubiquitinated or polyubiquitinated by attachment of a multiubiquitin chain linked through one of the internal lysine residues in ubiquitin [2]. K48-linked polyubiquitination is the canonical signal that targets proteins for degradation

by the 26S proteasome, whereas monoubiquitination and K63-linked polyubiquitination serve as regulatory signals to modulate protein activity, localization, and interactions [3,4]. Increasing evidence points to the critical importance of protein ubiquitination in the control of diverse cellular processes, from DNA repair and transcription regulation to vesicular trafficking and virus budding [4–6]. Moreover, dysregulated ubiqu-

Abbreviations

AD, Alzheimer's disease; APP, amyloid beta A4 protein; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GST, glutathione *S*-transferase; HA, hemagglutinin; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; IVEC, *in vitro* expression cloning; MVB, multivesicular body; RNP, ribonucleoprotein; siRNA, small interfering RNA; UIM, ubiquitin-interacting motif.

ubiquitination has been implicated in the pathogenesis of many human diseases, including cancer and neurodegenerative disorders [7]. Elucidation of the molecular mechanisms by which cells recognize and sort ubiquitinated proteins is thus essential for understanding ubiquitin signaling in both normal physiology and diseases.

The ubiquitin-interacting motif (UIM) is a conserved ubiquitin recognition module that was initially identified on the basis of the sequence homology to the ubiquitin-binding region of the S5a subunit of the 26S proteasome [8]. The UIM has a 20 amino acid consensus sequence $X\text{-Ac-Ac-Ac-Ac-}\Phi\text{-X-X-Ala-X-X-X-Ser-X-X-Ac-X-X-X-X}$, where Φ represents a large hydrophobic residue and Ac represents an acidic residue. UIMs are found in many proteins implicated in a variety of cellular processes, including endocytosis, endosome-to-lysosome trafficking, DNA repair, mRNA splicing, and neurodegeneration [8]. *In vitro* studies indicate that the UIM binds monoubiquitin and polyubiquitin chains [9–12]. Furthermore, UIM domains from different proteins bind polyubiquitin chains of varying lengths with different affinities [12], suggesting that different UIM domains may recognize distinct subsets of ubiquitinated proteins.

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is an early endosome-associated UIM-containing protein that plays a central role in controlling endosome-to-lysosome trafficking [13–16]. A major sorting decision in the endocytic pathway occurs at the early endosome, where membrane cargo proteins can be sorted to the recycling pathway for delivery to the cell surface or to luminal vesicles of multivesicular bodies (MVBs) for eventual degradation in the lysosome [6,16]. A sorting signal for cargo trafficking to the lysosomal pathway is the ubiquitination of cargo proteins. The UIM domain of Hrs has been shown to bind ubiquitin *in vitro* [9,11,14] and to facilitate the sorting of several ubiquitinated cargo proteins to the lysosomal pathway in mammalian cells [17] and yeast [10,18]. The Hrs UIM domain may also interact with ubiquitinated components of the endosomal trafficking machinery to regulate endosome-to-lysosome trafficking [6]. Recently, Hrs has been shown to preferentially bind K63-linked polyubiquitin chains [19]. Interestingly, the UIM domain is indispensable for monoubiquitination as well as phosphorylation of Hrs [9,12,20], raising the possibility that the Hrs UIM domain may bind E3 ubiquitin-protein ligase(s) and/or kinase(s). The identities of ubiquitinated cargo and other cellular proteins that are recognized by the Hrs UIM domain remain largely unknown.

In order to gain insight into the role of Hrs UIM-mediated ubiquitin signaling in cells, we performed a proteomic screen for Hrs UIM-interacting ubiquitinated proteins in human brain by using a combined *in vitro* expression cloning (IVEC) and glutathione *S*-transferase (GST) pull-down approach (Fig. 1). IVEC is a powerful screening method that combines biochemical analysis of radioactively labeled proteins with the ability to quickly isolate the corresponding cDNAs [21,22]. As compared to yeast two-hybrid screening, IVEC screening offers the advantage of studying direct interactions between two proteins *in vitro* [23], rather than indirect analysis of the interactions between fusion proteins inside the yeast nucleus. Moreover, our IVEC screening approach complements other proteomic screening strategies [24,25], which are often contaminated with secondary, nonspecific binding proteins.

Here, we report the identification of a set of proteins that are specifically recognized by the UIM domain of Hrs. Our results reveal the involvement of Hrs UIM-mediated protein interactions in the coordination of multiple steps in endosomal trafficking as well as in the regulation of cell signaling, cytoskeleton and membrane dynamics and other cellular processes.

Results

IVEC screen for proteins that are specifically recognized by the UIM domain of Hrs

To identify cellular targets of the Hrs UIM domain, we screened a human adult brain cDNA library for Hrs UIM-interacting proteins using an IVEC approach [21,22], which is summarized in Fig. 1. Pools of cDNAs (100 independent cDNA clones per pool) from the human brain library were *in vitro* transcribed and translated in the TNT Quick coupled transcription–translation reticulocyte lysate system in the presence of [^{35}S]methionine and ubiquitin to generate ^{35}S -labeled protein pools [23]. It has been well established that such a transcription–translation reticulocyte lysate system is capable of carrying out ubiquitination of *in vitro* translated proteins [23,26,27]. To determine whether the protein pools synthesized in our IVEC system are ubiquitinated, we performed immunoblot analysis with antibody against ubiquitin to examine the ubiquitination status of protein pools generated from the *in vitro* transcription–translation of human brain cDNA pools in the presence or absence of ubiquitin (Fig. 2B). We found that addition of ubiquitin to the *in vitro* transcription–translation reaction mixture dramatically increased the ubiquitination levels of *in vitro* translated protein pools, confirming that protein pools synthe-

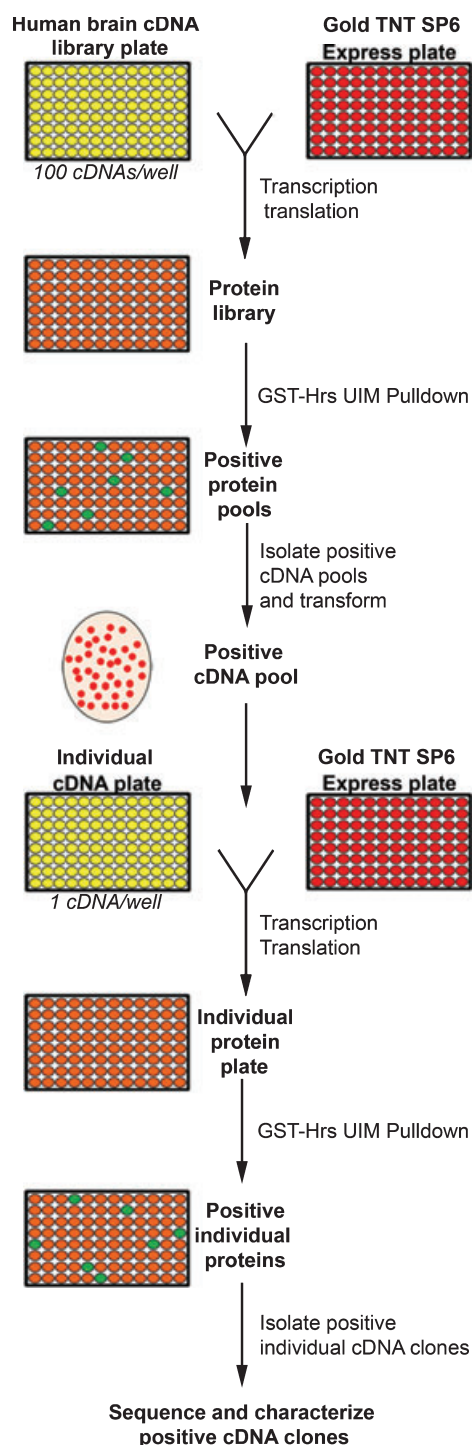


Fig. 1. Schematic illustrating the IVEC system used to identify and isolate cDNAs from human adult brain library that encode Hrs UIM-interacting proteins.

sized in our IVEC system are indeed ubiquitinated. The ^{35}S -labeled ubiquitinated protein pools were then tested for their ability to bind to a GST-fused UIM

domain of Hrs protein (Fig. 2A) in an *in vitro* binding assay. Figure 2C shows an example of six positive pools (pools 1, 2, 4, 5, 7, and 8) containing Hrs UIM-binding proteins isolated from the primary screen. From each of the positive pools, individual cDNA clones were isolated and subjected to a secondary screen in the same manner to identify positive cDNA clones encoding Hrs UIM-binding proteins (Fig. 2D). The specificity of the observed interactions was confirmed by the specific binding of the identified proteins to GST-Hrs UIM but not to GST control (Fig. 2E).

From the IVEC screen, we isolated 64 positive clones, which encode 48 proteins that are specifically recognized by the UIM domain of Hrs (Fig. 3 and Table 1). The specific binding of the identified proteins to the Hrs UIM domain suggests that these proteins may be ubiquitinated. In support of this notion, four of the identified proteins, APP [28], β -tubulin [29], Hsc70 [30,31], and MARK4 [32], have been previously shown to be ubiquitinated. However, the interaction of these proteins with the Hrs UIM domain has never been reported. The remaining 43 proteins have not previously been shown to be ubiquitinated or to bind to the Hrs UIM domain.

Classification of the identified Hrs UIM-interacting proteins

To shed light on the role of Hrs UIM-mediated protein interactions, we categorized the 48 proteins isolated from the IVEC screen according to functional predictions based on the available literature, gene ontology, and homology searches (Table 1). Classification of the identified Hrs UIM-interacting proteins according to their cellular localization (Fig. 3A) reveals that the majority of these proteins are integral membrane proteins (27%), membrane-associated proteins (21%), or cytosolic proteins (27%). We and others have shown that Hrs is associated with both early endosomal membrane and cytosolic fractions [13,33,34]. The localization of the majority of the identified Hrs UIM-binding proteins to the membrane and cytosol suggests that they are appropriately positioned to interact with Hrs in cells. In addition, we identified a number of proteins that could be classified as cytoskeletal (15%), which suggests that Hrs UIM-mediated ubiquitin signaling may have a role in regulation of cytoskeleton dynamics. The localization of only 4% and 6% of proteins could be classified as nuclear or unknown, respectively.

Dividing the identified Hrs UIM-interacting proteins on the basis of their functional classes (Fig. 3B) further suggests that the screen largely identified putative

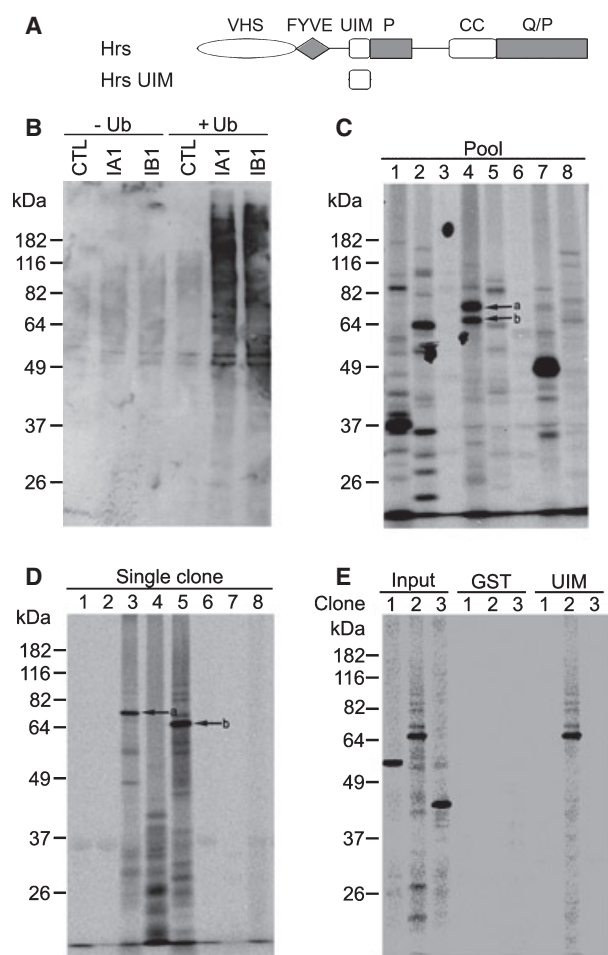


Fig. 2. IVEC screen for proteins that bind to the UIM domain of Hrs. (A) Domain structure of full-length Hrs (top) and the GST-fused Hrs UIM domain used in the IVEC screen (bottom). (B) Two cDNA pools, IA1 and IB1, containing 100 independent cDNA clones per pool from a human adult brain cDNA library, were *in vitro* transcribed and translated in the presence of cold methionine with or without ubiquitin. The control (CTL) reactions were carried out under the same conditions with no cDNAs added. The synthesized protein pools were analyzed by immunoblotting with antibody against ubiquitin. (C) Primary screen for positive pools containing Hrs UIM-binding proteins. Pools of cDNAs (100 independent cDNA clones per pool) from a human adult brain cDNA library were *in vitro* transcribed and translated in the presence of [³⁵S]methionine and ubiquitin and then subjected to a GST-Hrs UIM pull-down assay. Bound proteins were analyzed by SDS/PAGE. Autoradiography of gel samples was performed using a phosphorimager. Example of positive pools (pools 1, 2, 4, 5, 7, and 8) selected for secondary screen. The two bands labeled a and b in pool 4 represent distinct Hrs UIM-binding proteins, which would be individually isolated by secondary screen. (D) Secondary screen for isolation of individual positive cDNA clones encoding Hrs UIM-binding proteins. *In vitro* translated products from individual cDNA clones isolated from each of the positive pools were analyzed as described above for their ability to bind GST-Hrs UIM. Example of eight single clones isolated from pool 4, of which clones 3 and 5 are individual positive cDNA clones encoding Hrs UIM-binding proteins a and b indicated in (C). (E) Specificity of Hrs UIM domain binding. *In vitro* translated products from three isolated individual cDNA clones (Input) were incubated with immobilized GST-Hrs UIM fusion protein or GST control. Bound proteins were analyzed by SDS/PAGE and autoradiography. Clone 2 encodes a protein that specifically binds to GST-Hrs UIM but not to GST control, whereas clones 1 and 3 are negative interactors that bind neither to GST-Hrs UIM nor to GST control.

membrane cargo proteins and membrane cargo adaptor proteins (33%), consistent with the proposed function of Hrs in endosomal sorting and trafficking. The other major functional groups to which the Hrs UIM-interacting proteins belong include cell signaling (17%), metabolism (17%), vesicular trafficking (13%), and transport (10%), suggesting an interconnection between Hrs UIM-mediated ubiquitin signaling and these cellular processes.

Characterization of Munc18-1 and Hsc70 as Hrs UIM-interacting ubiquitinated proteins

The ability of the Hrs UIM domain to bind ubiquitin and ubiquitinated proteins has been well established [9,11,14,17,19]. Thus, the direct interaction between the Hrs UIM domain and each of the 48 proteins identified from our IVEC screen (Table 1) raises the possibility that these proteins are ubiquitinated in cells. To test this possibility, we used a well-established *in vivo* ubiquitination assay [35,36] to determine

the ubiquitination status of the identified Hrs UIM-interacting protein Munc18-1, a key regulator of Ca²⁺-dependent exocytosis [37], which has been previously unrecognized as a ubiquitinated protein. Lysates from HeLa cells expressing hemagglutinin (HA)-tagged ubiquitin and Myc-tagged Munc18-1 were subjected to immunoprecipitation with antibodies against Myc, followed by immunoblotting with antibodies against HA to detect HA-ubiquitin-conjugated Munc18-1 protein (Fig. 4A). We observed a prominent band around 82 kDa that may represent a diubiquitinated species of Munc18-1, as well as a higher molecular mass smear that may represent polyubiquitinated forms of Munc18-1. These results provide the first evidence that Munc18-1 is ubiquitinated *in vivo*, and support the notion that Hrs UIM-binding proteins isolated from our IVEC screen probably represent ubiquitinated proteins.

Next, we sought to determine whether ubiquitinated Munc18-1 is specifically recognized by Hrs UIM. *In vitro* binding assays were performed by incubating

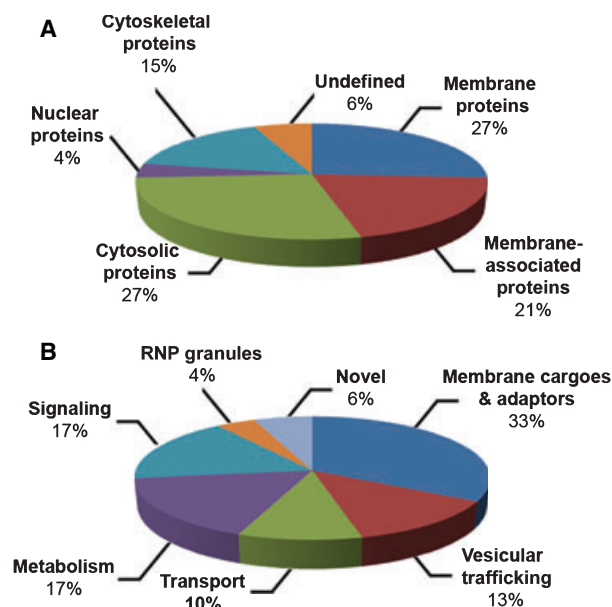


Fig. 3. Classification of the identified proteins according to cellular localization (A) and molecular function (B). The number of proteins in each category is expressed as the percentage of the total number of different proteins identified from the screen.

immobilized GST–Hrs UIM, full-length GST–Hrs, GST–HrsΔUIM, or GST control proteins (Fig. 5A) with soluble Myc-tagged Munc18-1 immunopurified from transfected HeLa cells. Bound proteins were probed with an antibody against ubiquitin and an antibody against Myc to detect ubiquitinated Munc18-1 and nonubiquitinated Munc18-1, respectively (Fig. 5B). We found that both GST-fused Hrs UIM domain and full-length Hrs selectively interacted with ubiquitinated Munc18-1 but not with nonubiquitinated Munc18-1 protein. The ability of Hrs to bind ubiquitinated Munc18-1 was dramatically reduced by the deletion of the UIM domain. Furthermore, the GST control did not pull down any detectable level of ubiquitinated or nonubiquitinated Munc18-1. Together, these results indicate that the Hrs UIM domain is both necessary and sufficient for binding Munc18-1 in a ubiquitin-dependent manner, and support the validity and specificity of our IVEC screen.

Our identification of 48 proteins as novel binding partners for the Hrs UIM domain has led to a number of interesting hypotheses. For example, previous studies have shown that Hrs is enriched with ubiquitinated cargo proteins in flat clathrin-coated microdomains of early endosomes [17,38,39]. These clathrin-coated microdomains have been proposed to play a role in endosomal sorting and retention of ubiquitinated cargo proteins [17,39]. The flat clathrin coat has to be

dissociated prior to endosomal invagination and budding of the MVB luminal vesicles [17,39], but the molecular machinery for the disassembly of the endosomal clathrin coat remains unknown. Hsc70 is a constitutively expressed member of the Hsp70 molecular chaperone family and has been shown to regulate clathrin uncoating processes [40,41]. Although Hsc70 is known to be ubiquitinated [30,31], it has been previously unrecognized as an Hrs-binding protein. Our identification of Hsc70 as an Hrs UIM-interacting protein raises an intriguing hypothesis that the Hrs UIM-mediated interaction recruits Hsc70 to endosomes for clathrin uncoating prior to the budding of MVB luminal vesicles. As a first step to test this hypothesis, we performed *in vivo* ubiquitination analysis to confirm that Hsc70 is indeed ubiquitinated in cells (Fig. 4B). Furthermore, we performed binding experiments and found that ubiquitinated Hsc70 specifically bound to GST–Hrs UIM and GST–Hrs, but not to GST–HrsΔUIM or the GST control (Fig. 5C), indicating that the Hrs UIM domain is both necessary and sufficient for binding ubiquitinated Hsc70. Our results showed that the Hrs UIM domain is unable to bind Hsc70 in the absence of ubiquitination, as GST–Hrs UIM did not pull down any detectable level of nonubiquitinated Hsc70 (Fig. 5C). Interestingly, our analysis revealed that the full-length Hrs was capable of interacting with nonubiquitinated Hsc70 and that this interaction was not affected by the deletion of the UIM domain (Fig. 5C), suggesting that the interaction of Hrs with nonubiquitinated Hsc70 is mediated by a binding site on Hrs that is located outside of its UIM domain.

Hsc70 is essential for ligand-induced epidermal growth factor receptor degradation

Next, we assessed the role of Hsc70 in the regulation of Hrs-mediated endosomal trafficking by using the epidermal growth factor (EGF) receptor (EGFR) as a cargo protein. Previous studies have shown that binding of EGF to the EGFR at the plasma membrane causes rapid internalization of the EGF–EGFR complex and subsequent sorting at the early endosome for delivery to the lysosome for degradation [42–44]. The role of Hrs-mediated early endosomal sorting in the regulation of EGF-induced EGFR degradation is well established; both the overexpression and the depletion of Hrs inhibit ligand-induced degradation of the EGFR [13,45]. Our identification of the interaction between Hsc70 and Hrs raises the possibility that Hsc70 may participate in the regulation of ligand-induced endocytic trafficking of the EGF–EGFR

Table 1. Hrs UIM-interacting proteins identified from the IVEC screen.

Gene	Protein name (alternative name)	Accession number
Membrane proteins		
<i>APP^a</i>	Amyloid beta (A4) protein	NP_958816
<i>APLP2</i>	Amyloid beta (A4) precursor-like protein 2	NP_001633
<i>ATP1A1</i>	Na ⁺ /K ⁺ -ATPase alpha 1 subunit	NP_000692
<i>ATP2A2</i>	ATPase, Ca ²⁺ transporting, slow twitch 2	NP_001672
<i>ARL6IP1</i>	ADP-ribosylation factor-like 6 interacting protein	NP_055976
<i>BSG</i>	Basigin	NP_940991
<i>C3orf1</i>	Hypothetical protein LOC51300	NP_057673
<i>FAM5C</i>	Family with sequence similarity 5, member C	NP_950252
<i>MEST</i>	Mesoderm specific transcript	NP_002393
<i>TMCC2</i>	Transmembrane and coiled-coil domain family 2	NP_055673
<i>TMEM49</i>	Transmembrane protein 49 (VMP1)	NP_112200
<i>UNC84B</i>	Unc-84 homolog B (Rab5IP)	NP_056189
Membrane protein-associated adaptor proteins		
<i>AHCYL1</i>	S-adenosylhomocysteine hydrolase-like 1 (IRBIT)	NP_006612
<i>CASK^b</i>	Calcium/calmodulin-dependent serine protein kinase	NP_003679
<i>TJP2</i>	Tight junction protein 2 (ZO-2)	NP_963923
<i>TRAP1</i>	TNF receptor-associated protein 1	NP_057376
Vesicular trafficking		
<i>GGA2^c</i>	ADP-ribosylation factor binding protein 2	NP_055859
<i>HSPA8^a</i>	Heat shock 70 kDa protein 8 (Hsc70)	NP_006588
<i>MAP3K10^b</i>	Mitogen-activated protein kinase 10 (MLK2)	NP_002437
<i>STXBP1</i>	Syntaxin-binding protein 1 (Munc18-1)	NP_003156
<i>SCRN1</i>	Secernin 1	NP_055581
Cytoskeleton and cytoskeleton-dependent transport		
<i>CRMP1</i>	Collapsin response mediator protein 1	NP_001304
<i>DCTN2</i>	Dynactin 2 (dynamitin)	NP_006391
<i>GFAP^a</i>	Glial fibrillary acidic protein	NP_002046
<i>MARK4^a</i>	MAP/microtubule affinity-regulating kinase 4	NP_113605
<i>PPP1R16A</i>	Protein phosphatase 1, regulator (inhibitor) subunit 16A (MYPT3)	NP_116291
<i>RHOBTB3</i>	Rho-related BTB domain containing 3	NP_055714
<i>TUBB^a</i>	Tubulin, beta	NP_821133
<i>TUBB2A</i>	Tubulin, beta 2	NP_001060
Cell signaling		
<i>ESRRG</i>	Estrogen-related receptor gamma (ERR3)	NP_996317
<i>ILKAP</i>	Integrin-linked kinase-associated protein phosphatase 2C	NP_789769
<i>PPP1R7</i>	Protein phosphatase 1 regulatory subunit 7	NP_002703
<i>RSU1</i>	Ras suppressor protein 1	NP_036557
<i>UBA1</i>	Ubiquitin-activating enzyme E1	NP_003325
Metabolism		
<i>ACLY</i>	ATP citrate lyase	NP_942127
<i>CBS</i>	Cystathionine-beta-synthase	NP_000062
<i>DECR</i>	2,4-Dienoyl CoA reductase 1	NP_001350
<i>MAT2A</i>	Methionine adenosyltransferase II alpha	NP_005902
<i>MTHFD1L</i>	C1 tetrahydrofolate synthase	NP_056255
<i>OSBPL5</i>	Oxysterol-binding protein-like protein 5	NP_065947
<i>PLD3</i>	Phospholipase D family, member 3	NP_036400
<i>PFKM</i>	Phosphofructokinase, muscle	NP_000280
Ribonucleoprotein granules		
<i>HNRPDL</i>	Heterogeneous nuclear ribonucleoprotein D-like	NP_112740
<i>RPS3A</i>	Ribosomal protein S3a	NP_000997
<i>SF3B3</i>	Splicing factor 3b, subunit 3	NP_036558
Novel proteins		
<i>LOC349114</i>	Hypothetical protein LOC349114	Q8N836
<i>PTCD3</i>	Pentatricopeptide repeat domain 3	NP_060422
<i>ZNF302</i>	Zinc finger protein 302	NP_060913

^a Known to be ubiquitinated. ^b Interacts with an E2 or E3, but is not known to be ubiquitinated. ^c Thought to be ubiquitinated on the basis of similarity.

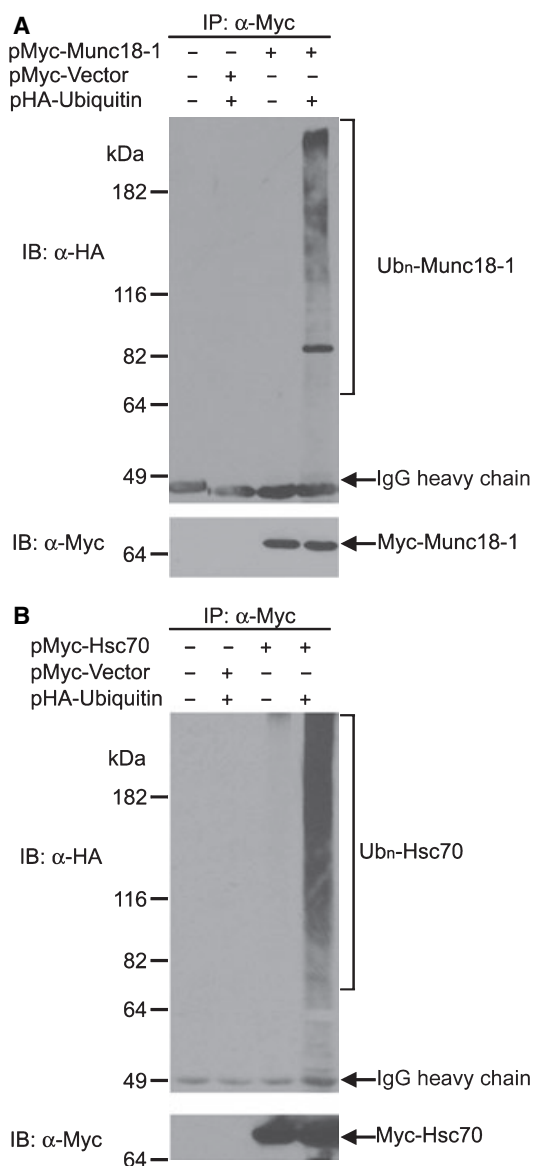


Fig. 4. Munc18-1 and Hsc70 are ubiquitinated in cell-based assays. (A) HeLa cells were transfected with the indicated plasmids and treated with proteasome inhibitor MG132 for 8 h before harvest. Cell lysates were subjected to immunoprecipitation with antibody against Myc, followed by immunoblotting with antibody against HA to detect HA-tagged ubiquitin conjugated to Munc18-1 (upper panel). The blot was then reprobed with antibody against Myc to detect Myc-tagged Munc18-1 protein (lower panel). (B) *In vivo* ubiquitination of Hsc70 was analyzed using the same assay as described above. Data are representative of at least three independent experiments.

complex to the lysosome for degradation. To test this possibility, we examined the effect of depleting Hsc70 through small interfering RNA (siRNA) on EGF-induced EGFR degradation. For selective depletion of endogenous Hsc70, we used two distinct siRNA

duplexes, Hsc70 siRNA-1 and Hsc70 siRNA-2, which specifically target different regions of the Hsc70 mRNA. Immunoblot analysis confirmed that Hsc70 siRNA-1 (data not shown) and Hsc70 siRNA-2 (Fig. 6A) both specifically inhibited the expression of endogenous Hsc70, but not EEA1.

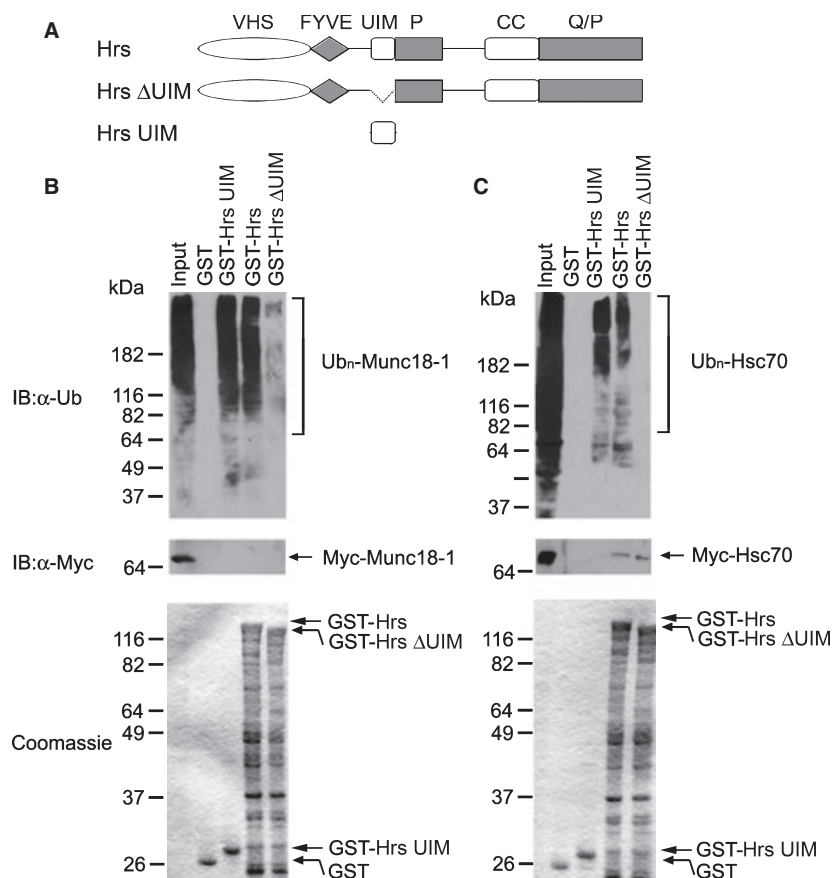
Next, we examined the effect of siRNA-mediated knockdown of Hsc70 expression on the uptake and degradation of [125 I]EGF in HeLa cells. We found that depletion of Hsc70 by Hsc70 siRNA-2 (Fig. 6B) had no statistically significant effect on [125 I]EGF internalization. As shown in Fig. 6C, we observed a statistically significant ($P < 0.05$) decrease in [125 I]EGF degradation in Hsc70 siRNA-2 ($41.9 \pm 7.5\%$, $n = 4$) transfected HeLa cells as compared to the untransfected controls ($73.6 \pm 2.2\%$, $n = 4$) and control siRNA transfected cells ($73.6 \pm 7.3\%$, $n = 4$). Similar effects were observed when using Hsc70 siRNA-1. Together, these data provide strong evidence supporting a functional role for Hsc70 in the regulation of the trafficking of internalized EGF-EGFR complexes to the lysosome for degradation.

Discussion

The present study represents the first large-scale unbiased screen for candidate proteins that are specifically recognized by the UIM domain of Hrs. Our screening results demonstrate that the IVEC screen for identification of Hrs UIM-interacting proteins is highly specific, as out of 48 000 independent human brain cDNA clones screened, we only isolated 64 positive clones corresponding to 48 distinct proteins. Furthermore, among the identified proteins, we did not find any proteins that are exclusively localized to the extracellular matrix. The validity of our IVEC screen is supported by our *in vivo* ubiquitination assays showing that two identified Hrs UIM-interacting proteins, Munc18-1 and Hsc70, are indeed ubiquitinated in cells. Furthermore, the results of our deletion mutagenesis and binding experiments clearly demonstrate that the Hrs UIM domain is both necessary and sufficient for selective interaction with the ubiquitinated forms of Munc18-1 and Hsc70 but not with the nonubiquitinated forms of these proteins. Together, these data strongly suggest that the Hrs UIM-interacting proteins identified in our IVEC screen (Table 1) are likely to be ubiquitinated proteins.

The current model for Hrs UIM domain function is that the Hrs UIM domain binds ubiquitinated membrane cargo proteins at early endosomes, thereby facilitating the sorting of these proteins to the lysosomal pathway [6,15,16]. In support of this model,

Fig. 5. Hrs directly binds ubiquitinated Munc18-1 or ubiquitinated Hsc70 in a UIM-dependent manner. (A) Domain structure of GST-Hrs fusion proteins. (B) Soluble immunopurified Myc-tagged Munc18-1 (input) was incubated with similar amounts of immobilized GST or GST-Hrs fusion proteins (lower panel). Immunoblot analysis of bound proteins with antibody against ubiquitin (upper panel) and antibody against Myc (middle panel) reveals a UIM-dependent interaction of Hrs with ubiquitinated Munc18-1 but not with nonubiquitinated Munc18-1 protein. (C) Soluble immunopurified Myc-tagged Hsc70 (input) was incubated with similar amounts of immobilized GST or GST-Hrs fusion proteins (lower panel). Immunoblot analysis of bound proteins with antibody against ubiquitin (upper panel) and antibody against Myc (middle panel) reveals that Hrs binds ubiquitinated Hsc70 and nonubiquitinated Hsc70 protein through different domains. The immunopurified Myc-tagged, ubiquitinated and nonubiquitinated forms of Munc18-1 or Hsc70 in the input lane were detected by immunoblotting with antibody against ubiquitin and antibody against Myc, respectively, but their amounts were too low for detection by the Coomassie stain.



we identified nine known and three novel membrane proteins as Hrs UIM-interacting proteins (Table 1), which probably represent endosomal cargo proteins that undergo ubiquitination-dependent sorting by Hrs. Among the Hrs UIM-interacting membrane proteins, we identified amyloid beta A4 protein (APP) and the related APP-like protein 2. Mutations in the *APP* gene are associated with Alzheimer's disease (AD) [46]. Previous studies have shown that APP localizes to endosomes [47] and that APP is ubiquitinated [28]. Our finding that the Hrs UIM domain binds to APP is of particular interest, given the increasing evidence that endosomal abnormalities, specifically enlarged early endosomes, precede the appearance of symptoms in AD [48]. Our study provides the first report of an interaction between a component of the endosomal sorting machinery and APP and suggests that aberrant Hrs-mediated endosomal sorting of APP may be involved in AD pathogenesis.

Our IVEC screen results support an additional role for the Hrs UIM domain in the sorting of nonubiquitinated membrane cargo proteins to the lysosomal pathway. Recent studies have revealed that not all membrane cargo proteins require ubiquitination for

trafficking to lysosomes [49] and that 'ubiquitination-independent' cargo trafficking also requires Hrs for sorting to lysosomes [50]. The mechanism underlying Hrs-dependent endosome-to-lysosome trafficking of nonubiquitinated membrane cargo protein is not understood. Interestingly, our identification of four membrane protein-associated adaptor proteins, CASK [51], ZO-2 [52], IRBIT [53], and TRAP1 [54], as putative ubiquitinated proteins recognized by the Hrs UIM domain raises an intriguing possibility that the ubiquitination of adaptor proteins may act as a sorting signal for targeting their associated membrane proteins to the lysosomal pathway.

In addition to membrane cargo and adaptor proteins, we identified five proteins that function in vesicular trafficking (Table 1), including GGA2 and MLK2. GGA2 belongs to a family of Arf-dependent adaptors that bind clathrin and mediate the sorting of cargo proteins at the trans-Golgi network for delivery to endosomes [55]. Recent evidence indicates that GGA proteins function not only at the trans-Golgi network, but also at early endosomes to facilitate the transport of endosomal cargo proteins into the MVB [56]. MLK2 is a protein kinase that functions in the

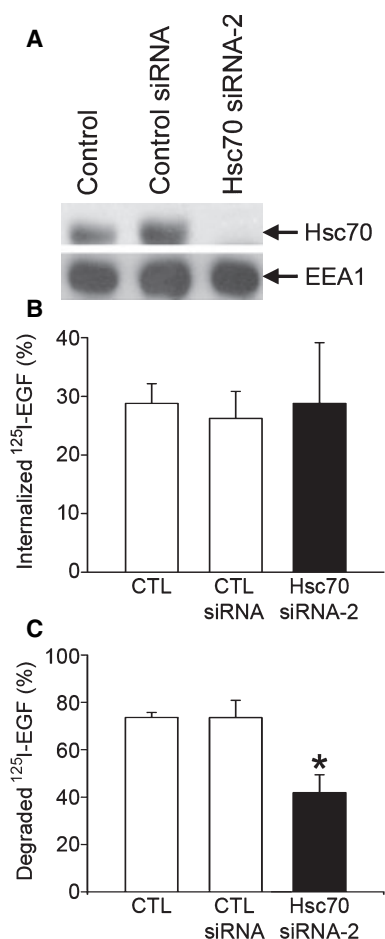


Fig. 6. Hsc70 knockdown inhibits EGF-induced EGFR degradation. (A) Equal amounts of proteins from HeLa cell lysates transfected with the indicated siRNA were analyzed by immunoblotting with antibodies against Hsc70 and EEA1. (B) HeLa cells transfected with the indicated siRNAs were incubated with [^{125}I]EGF for 10 min at 37 °C. The internalized [^{125}I]EGF is expressed as a percentage of the initially bound [^{125}I]EGF. (C) HeLa cells transfected with the indicated siRNAs were allowed to internalize [^{125}I]EGF for 10 min, and then chased for 1 h at 37 °C. The degraded [^{125}I]EGF is expressed as a percentage of the initially internalized [^{125}I]EGF. Data represent mean \pm standard error of the mean from three independent experiments. The asterisks indicate a statistically significant difference ($P < 0.05$) from the control siRNA-transfected cells.

stress-activated Jun N-terminal kinase signaling pathway and has been shown to bind clathrin via its C-terminal clathrin box motif and regulate clathrin-coated vesicle trafficking [57]. Interestingly, Hrs also contains a C-terminal clathrin box motif that binds clathrin, and the ability of Hrs to bind clathrin is essential for the formation of Hrs–clathrin sorting microdomains on early endosomes [17,38,39,58]. The identification of GGA2 and MLK2 as Hrs UIM-interacting ubiquitinated proteins suggests that these two proteins may

work in concert with Hrs in the clathrin-dependent endosomal sorting and retention process.

As clathrin is not incorporated into MVB luminal vesicles, the flat clathrin coat on the early endosome has to be dissociated prior to the budding of the luminal vesicles [17,39]. The molecular machinery for the dissociation of the endosomal clathrin coat remains undefined. In this study, we identified the clathrin-uncoating ATPase Hsc70 as an Hrs UIM-interacting ubiquitinated protein, and provided evidence that Hsc70 is an essential component of the machinery that regulates Hrs-mediated endosome-to-lysosome trafficking of internalized EGF–EGFR complexes. Our findings support the idea that Hsc70 is part of the clathrin-uncoating machinery at early endosomes and that loss of Hsc70 inhibits this uncoating process and subsequent delivery of cargo proteins to the MVB pathway for degradation in the lysosome.

The other two identified proteins in the vesicular trafficking category are Munc18-1, an essential component of the molecular machinery for synaptic vesicle exocytosis [37,59], and secernin 1, a cytosolic protein involved in the regulation of exocytosis from mast cells [60]. Our identification of these two proteins as Hrs UIM-binding partners suggests a role for Hrs in the regulation of Ca^{2+} -dependent exocytosis. Consistent with this role, we and others have previously reported a functional interaction between Hrs and SNAP-25, a vesicular SNARE protein involved in synaptic vesicle exocytosis [33,61–63]. Our results obtained from the present study provide the first evidence that Munc18-1 is ubiquitinated in cells, and suggest that Munc18-1 ubiquitination and Hrs UIM-mediated ubiquitin signaling may regulate the exocytosis process.

Our IVEC screen also resulted in the isolation of eight proteins that function in the regulation of microtubule, actin and intermediate filament cytoskeletal networks and their associated motors (Table 1). Microtubules are dynamic protein filaments that serve as tracks for regulated movement and intracellular positioning of organelles, including endosomes [64]. The identification of β -tubulins, MARK4 [32,65,66] and dynactin 2 [67] as Hrs UIM-interacting proteins suggests a previously unrecognized role of Hrs in regulating microtubule dynamics and microtubule-based transport of endosomes. The interaction of the Hrs UIM domain with dynactin 2 is of particular interest, because it provides a mechanism for loading endosomes onto microtubules and converting them to a motile pool. In addition to microtubules, the dynamics of the actin cytoskeleton and intermediate filaments have also been implicated in the regulation of endosomal trafficking [64,68,69]. Our identification of

RhoBTB3 [70], CRMP-1 [71], MYPT3 [72,73] and GFAP [74,75] as Hrs UIM-interacting proteins suggests a role for Hrs in the coordinated regulation of actin dynamics, intermediate filament dynamics, and endosomal trafficking.

We and other laboratories have shown that Hrs exists in both cytosolic and endosomal membrane-associated pools [13,33,34]. Our screening results (Table 1) raise the possibility that, in addition to endosome-associated Hrs UIM-mediated ubiquitin signaling, the cytosolic Hrs UIM domain may play a role in the regulation of multiple cellular processes, including exocytosis, signal transduction, transport of ribonucleoprotein (RNP) granules, and various metabolic processes. The wealth of data and interesting hypotheses generated from this study provide a basis for further studies to elucidate the molecular mechanisms underlying Hrs UIM-mediated ubiquitin signaling in cells.

Experimental procedures

Expression constructs and antibodies

Standard molecular biological techniques were used to generate pGST–Hrs UIM, which directs the expression of an N-terminal GST-tagged Hrs UIM domain corresponding to amino acids 251–286 of rat Hrs [33]. The pGST–Hrs UIM expression construct was sequenced to ensure that the fusion was in the correct reading frame and there were no unwanted changes in the codons. The pHA–ubiquitin [35], pGST–Hrs, and pGST–HrsΔUIM [76] constructs have been described previously. The pMyc–Hsc70 and pMyc–Munc18-1 plasmids were obtained as generous gifts from C. Patterson (University of North Carolina at Chapel Hill, NC, USA) and T. Südhof (University of Texas Southwestern, TX, USA), respectively. Antibodies used in this study include the following: anti-HA (3F10; Boehringer Mannheim, Mannheim, Germany; HA.11, Covance, Princeton, NJ, USA), anti-Hsc70 (Stressgen, Ann Arbor, MI, USA), anti-Myc (9E10.3; Neomarkers, Fremont, CA, USA), anti-ubiquitin (P4G7 and FL76; Covance), anti-EEA1 (BD Transduction Laboratories, San Jose, CA, USA), and secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch Labs, Inc., West Grove, PA, USA).

IVEC screen for Hrs UIM-interacting proteins

For identification of ubiquitinated proteins that bind to the UIM domain of Hrs, an IVEC screen (Fig. 1) of a human adult brain cDNA library was performed using the Proteo-Link IVEC system (Promega Corporation, Madison, WI, USA). The brain library cDNAs in a 96-well format with 100 cDNAs per well were *in vitro* transcribed and translated in the Gold TNT Quick coupled transcription–translation

reticulocyte lysate system (Gold TNT SP6 Express 96-well plate) in the presence of [³⁵S]methionine and ubiquitin as described previously [23]. The obtained protein pools were incubated at 4 °C for 2 h in binding buffer with GST–Hrs UIM fusion protein (Fig. 2A, bottom) or GST control immobilized on glutathione–agarose beads. After extensive washes with washing buffer, bound proteins were eluted by boiling in the Laemmli sample buffer, and analyzed by SDS/PAGE. Autoradiography of gel samples was performed using a phosphorimager. For each positive protein pool, the corresponding cDNA pool was progressively subdivided and re-examined in the same manner until individual positive cDNA clones were isolated [22]. Positive clones were then analyzed by DNA sequencing and by BLAST searches for sequence homology in the NCBI database. Putative transmembrane proteins were identified using both of the predictive HMMTOP servers [77,78].

Classification of Hrs UIM-interacting proteins

The identified Hrs UIM-interacting proteins were classified according to their subcellular localization and molecular function as determined on the basis of the available literature, gene ontology, and homology searches. The percentage of proteins in each category was calculated by normalizing the number of proteins in each group to the total number of different proteins identified from the IVEC screen.

In vivo ubiquitination assays

In vivo ubiquitination assays were performed as described previously [35,36]. Briefly, HeLa cells were transfected with pHA–ubiquitin in combination with pMyc–Munc18-1 or pMyc–Hsc70, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were incubated for 8 h with proteasome inhibitor MG132 (20 μM in dimethylsulfoxide). The cells were then lysed, and an equal amount of protein from each lysate was subjected to denaturing immunoprecipitation using antibodies against Myc. Immunoprecipitates were analyzed by SDS/PAGE, followed by immunoblotting with an antibody against HA to detect HA–ubiquitin conjugated to Munc18-1 or Hsc70.

Ubiquitin binding assays

GST–Hrs fusion proteins (~200 pmol) or GST control immobilized on glutathione–agarose beads were incubated at 4 °C for 2 h in binding buffer (25 mM Tris, pH 7.5, 125 mM NaCl, 0.1% IGEPAL CA630) with ubiquitinated Munc18-1 or Hsc70 immunopurified from transfected HeLa cells [36,79]. After extensive washes, bound proteins were eluted by boiling in the Laemmli sample buffer, and analyzed by SDS/PAGE and immunoblotting [80].

siRNA transfection

Two siRNAs (Dharmacon, Lafayette, CO, USA) were generated against the human Hsc70 mRNA sequences 3'-GG AGGUGUCUUCUAUGGUUUU-5' and 3'-GAACAAG AGAGCUGUAAGAUU-5', called Hsc70 siRNA-1 and siRNA-2, respectively. In addition, a control siRNA with no known mammalian homology (siCONTROL Non-Targeting siRNA #1, Dharmacon) was used as a negative control. HeLa cells were transfected with the indicated siRNA (50 nM), using the *TransIT siQUEST* (Mirus, Madison, WI, USA) reagent according to the manufacturer's instructions. At 72 h post-transfection, cells were lysed, and an equal amount of protein from each lysate was subjected to SDS/PAGE and immunoblotting with antibodies against Hsc70 and EEA1.

[¹²⁵I]EGF internalization and degradation assays

For measurement of [¹²⁵I]EGF internalization, cells were serum starved for 2 h, and then incubated on ice with ~20 ng·mL⁻¹ [¹²⁵I]EGF (MP Biochemicals, Solon, OH, USA) in binding buffer (1% BSA in serum-free DMEM). Cells were then washed with cold binding buffer, and either lysed immediately to measure the initially bound [¹²⁵I]EGF, or transferred to 37 °C for 10 min. After washing of cells with acid wash (0.5 M NaCl, 0.2 M acetic acid, pH 2.8) on ice, the internalized [¹²⁵I]EGF was measured as previously described [81,82] and expressed as a percentage of the initially bound [¹²⁵I]EGF. For measurement of [¹²⁵I]EGF degradation after internalization, cells were chased in serum-free DMEM containing 1.5 µg·mL⁻¹ EGF and 1% BSA at 37 °C for 60 min. Degraded [¹²⁵I]EGF was measured as previously described [81,82] and expressed as a percentage of the initially internalized [¹²⁵I]EGF. Data are presented as the mean (± SEM) and are representative of at least three independent experiments.

Acknowledgements

We thank T. Südhof and C. Patterson for providing the expression constructs for Munc18-1 and Hsc70, respectively. J. W. Pridgeon was supported by National Institute of Neurological Disorders and Stroke Training Grant T32NS007480. This work was supported by grants from the National Institutes of Health (NS047575 and GM082828 to L. Li and NS050650 to L.-S. Chin).

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